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The role of hypochlorous acid (HOCl)
in sodium chloride-induced inhibition
of virus replication

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Master of Science by Research in Infections Disease

The University of Edinburgh

2016

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Abstract

There is evidence to suggest hypertonic saline (NaCl solution) could improve symptoms and reduce hospital stay in patients with viral respiratory tract infections (VRTI) such as bronchiolitis and chronic rhinosinusitis. According to current theory the effect of NaCl in VRTI treatment results from its mucolytic effect where saline helps clear the airway secretions by reducing the viscosity and elasticity of mucus gel. However, this mechanism remains controversial and few studies have been done to look at the potential antiviral effect of NaCl. Here we demonstrate that NaCl could inhibit the replication of several DNA and RNA viruses including varicella zoster virus (VZV) and respiratory syncytial virus (RSV). We also found that other sodium salt and chloride salt such as NaH_2PO_4 and NH_4Cl can also inhibit viral replication, suggesting that both sodium and chloride ions contribute to the antiviral effect by NaCl. Furthermore, we show that HeLa cells could produce HOCl as an immune response during viral infection and additional NaCl could increase the level of HOCl production. HOCl has been shown to be produced by neutrophil to neutralize pathogenic infection. Our results suggest that the NaCl has a broad antiviral effect which depends on the promotion of HOCl production in epithelial cells. This research indicates that NaCl could be a safe, effective and cheap antiviral agent for viral infection, especially VRTI and epithelial viral infection. We also reveal that HOCl production is a new antiviral mechanism in epithelial cells and it could be used to regulate viral replication.

Layman's abstract

There is evidence to suggest that hypertonic saline (NaCl solution), known as salt water, could improve symptoms and reduce hospital stay in patients with viral respiratory tract infections (VRTI) such as bronchiolitis and chronic rhinosinusitis. According to current theory the effect of NaCl in VRTI treatment results from its mucolytic effect where saline could thin down the airway secretions which finally lead to secretion clearance. However, this mechanism remains controversial and few studies have been done to look at if NaCl could inhibit viral replication in VRTI. Here we demonstrate that NaCl could inhibit the replication of several viruses including varicella zoster virus (VZV) which cause chicken pox and respiratory syncytia virus (RSV) which cause human respiratory infections. We found other salts such as NH_4Cl and NaH_2PO_4 could also inhibit viral replication. Because these salts provide sodium ion and chloride ion respectively, this result suggests that both sodium and chloride ions contribute to the antiviral effect by NaCl. Furthermore, we show that viral infection could induce intracellular hypochlorous acid (HOCl) production as an antiviral immune response in epithelial cells. HOCl is the main active substance in household bleach and could inactivate viruses. The additional NaCl could increase the level of such HOCl production, which lead to a reduction of viral replication. This research indicates that NaCl could be a safe, effective and cheap antiviral agent for viral infection, especially VRTI and epithelial viral infection. We also reveal that HOCl production is a new antiviral mechanism in epithelial cells and it could be used to regulate viral replication.

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Abbreviations

293T	Human kidney epithelial cells
3T3	Mouse embryo fibroblast cells
A549	Human lung epithelial cells
AFC	Alveolar fluid clearance
BHH	Benzamil
BHK-21	Hamster kidney fibroblast cells
CaCC	Calcium activated chloride channels
CFTR	Cystic fibrosis transmembrane conductance regulator
ClC	Voltage gated chloride channels
CS	Calf serum
Ct	Cycle threshold
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Chemiluminescence
EMEM	Eagle's minimum essential medium
FCS	Fetal calf serum
GFP	Green fluorescent protein
HeLa	Human cervix epithelial cells
HL-60	Human promyelocytic leukemia cells
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
HSV-1	Herpes simplex virus 1
IAV	Influenza A virus
IFN	Interferon
IgG	Immunoglobulin G
LGCC	Ligand gated chloride channels
LGSC	Ligand-gated sodium channels
MeWo	Human skin fibroblast cells

MOI	Multiplicity of infection
MPO	Myeloperoxidase
mRNA	Messenger RNA
NEAA	Non-essential amino acids
NHS	National health service
NP	Nucleoprotein
NPPB	5-Nitro-2-(3-phenylpropylamino) benzoic acid
P/S	Penicillin and Streptomycin
PBS	Phosphate buffered saline
PKC	Protein kinase C
qRT-PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxidant species
RSV	Respiratory syncytial virus
RTI	Respiratory tract infection
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNAs
TBS	Tris-buffered saline
URTI	Upper tract respiratory infection
Vero	Monkey kidney epithelial cells
VGSC	Voltage-gate sodium channels
VRTI	Viral respiratory tract infection
VZV	Varicella zoster virus

1. Introduction

1.1 The application of saline in viral respiratory tract infection (VRTI)

1.1.1 General introduction of VRTI

Respiratory tract infection (RTI) is one of the most common infections in humans causing significant morbidity and mortality due to its high attack rate. Most upper RTIs (URTIs) are caused by virus such as respiratory syncytial virus (RSV), coronaviruses, parainfluenza viruses, influenza viruses and adenoviruses with fewer than 10% URTIs of bacterial origin. Patients with a viral RTI (VRTI) have a median duration of 7.4 days, while 25% of the VRTI cases last 2 weeks. VRTI is also a leading cause of death among children younger than 5 years. Individuals with asthma, sinusitis, otitis media, immunodeficiency or any other clinical risk factors suffer more from VRTI and up to 45% of acute asthma exacerbations are thought to be caused by VRTI. (A. Mark Fendrick 2003).

Aside from the morbidity and related mortality suffering, VRTI also causes great economic burden. In the United States, non-influenza-related RTI caused approximately \$17 billion on direct medical costs and \$22.5 billion indirect costs which make up 2.9% of the annual national health expenditures in the United States in 2000.(A. Mark Fendrick 2003) In the UK, URTI is the most common reason for patients to visit their general practitioners, which causes burden on the public health service. (Easton & Saxena 2010)

Currently treatment for URTI is mainly aimed at reducing symptoms by applying antipyretic and analgesic drugs, mucolytics, expectorants and decongestants. However, 33% of children with URTI received antibiotic prescription in UK, despite the fact that antibiotics usage does not affect viral URTI but contribute to the development of antibiotic resistance. (Easton & Saxena 2010) The drug usage in RTI brings up NHS spending where annual prescribing costs for acute cough alone exceed £15 million. (NICE 2008) An economic and effective agent, preferably antiviral, is urged to treat

URTI infections.

1.1.2 The application of hypertonic saline in URTI infection

Saline is a cheap and well known agent used in URTI to ease symptoms by gargling or nasal irrigation. Research shows that the use of 3% saline reduced the hospital stay of infants with viral bronchiolitis (Teunissen et al. 2014) and saline nasal irrigation improved the symptoms of chronic rhinosinusitis. (Bachmann et al. 2000) Another summary by Cochrane group reviews 11 randomised trials involving 1090 infants with mild to moderate bronchiolitis. Review suggests that nebulised hypertonic saline could lead to 1.2 days reduction of hospital stay as well as improvement on clinical score. This study also found that there were no significant adverse effects noted during the application of hypertonic saline (Table 1). (Zhang et al. 2011) These results suggest that use of hypertonic saline is an effective treatment adjunct with no significant adverse effect.

Table 1. Summary of the effects of saline in different respiratory diseases

Researcher	RTI type	Patients (mean age)	Method	Outcome
Kuzik BA et. al	Viral bronchiolitis	96 infants (4.7 months)	Inhalation of Nebulised 3% saline in addition to routine therapy	26% reduction of hospital length of stay
Avigdor M et. al	Viral bronchiolitis	52 infants (2.9 months)	Inhalation of 4ml nebulised 3% saline with 1.5mg epinephrine	25% reduction of hospital stay and improvement in the clinical severity scores
Sarrell EM et. al	Viral bronchiolitis	65 infants (12.5 months)	Inhalation of 2ml nebulised 3% saline solution with 55mg terbutaline	Improvement in the clinical severity scores
Tal. G Et. al	Viral bronchiolitis	41 infants (2.6 months)	Inhalation of 4ml nubulised 3% saline with 1.5mg epinephrine	26% reduction of hospital stay and improvement in the clinical scores.
Harvey R	Chronic rhinosinusitis	1090 infants	Nasal saline irrigations	Improve symptoms of chronic rhinosinusitis

1.2 Antiviral effects of NaCl and HOCl

1.2.1 The effect of NaCl on virus infectivity

The mechanism of effect by saline in URTI infection is still not fully illustrated. Current hypothesis is that the saline inhalation increases the osmolarity of the airway surface, which results in an increase of mucociliary clearance. (Daviskas et al. 1996; Wills et al. 1997) Nasal irrigation with hypertonic saline was also shown to improve mucociliary transit. (Bachmann et al. 2000) However, most of these hypotheses are mostly discussed in the area of cystic fibrosis, where patients suffered from the loss of mucociliary, but not the area of VRTI. The mechanism of mucociliary clearance is poorly demonstrated and few links are found between mucociliary clearance and improve of URTI symptoms. An alternative hypothesis is that saline could directly inhibit viral replication in VRTI treatment. The antiviral effect of NaCl has been demonstrated with mengo virus. Study result found that NaCl could significantly reduce mengo virus infectivity from 15mM up to 150mM. Inactivation of virus could be found on other chloride salts at 150mM (chloride ion concentration) but no significant inactivation was found in sodium salts. In sodium halide salts, inactivation of the virus can be found extensively in bromide, somewhat less in iodide. (Spir 1960)

We could conclude that sodium ion itself is not a sufficient condition for inactivation but halide ions, especially chloride ions, affect the virus infectivity.

1.2.2 The role of HOCl in immune system

A possible explanation for the antiviral effect of NaCl is that chloride ions are utilized by immune system to synthesise active substances such as HOCl, which could eliminate viral replication. Human immune system developed multiple immune strategies in response of viral infection. These strategies include adaptive immune response that targets at specific pathogens by lymphocytes (Zeev Pancer and Max D. Cooper 2006), and innate immune system which non-specifically prevents infection

by surface barriers, inflammation, complement system and cellular barriers. (Medzhitov 2007) As part of the cellular barrier, phagocytes such as macrophages and neutrophils cells can kill pathogens by realising hypochlorous acid (HOCl). (Zgliczynski 1976) HOCl is an active oxidizer that reacts with a large variety of molecules including fatty acid and proteins. (Jaimes et al. 2015) The biological reactivity of HOCl results from its oxidative degradation effect, where proteins are degraded and DNA synthesis is significantly affected. (Albrich et al. 1981) People have found a reduction of Influenza A virus (IAV) and rhinovirus, which are common VRTI pathogens, *in vitro* when culture system was exposed to 3.5 ppm HOCl solution. (Sanekata 2010) In respiratory infection, HOCl is produced *in vivo* by macrophages to digest viral protein. (Inoguchi 2003)

The production of HOCl by macrophages and neutrophil cells depend on the enzyme myeloperoxidase (MPO). MPO is a 150-kDa protein consisting of two 15-kDa light chains and two variable-weight heavy chains which catalyse the conversions of chloride ions and hydrogen peroxide to hypochlorous acid (or other equivalent halide ions to hypohalid acid) and hydrogen peroxide. (Figure 1)

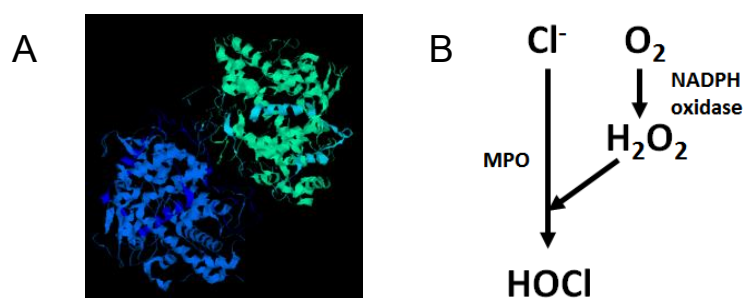


Figure 1. The structure of MPO (a) and the pathway of HOCl production (b). The oxygen is converted to hydrogen peroxide by NADPH oxidase and chloride ions react with peroxide ions to form HOCl, catalysed by MPO. Figure 1 A from Blair (Blair-Johnson et al. 2001)

1.3 Hypothesis and aims

We hypothesize that viral replication could inducing intracellular HOCl as an innate antiviral immune response, and NaCl could inhibit virus replication by providing chloride ions, which enter the cells through chloride ion channels, as substrate to

increase HOCl production.

To test this hypothesis, we aim to explore:

1. Effect of NaCl on viral replication such as RSV, IAV, and VZV.
2. The effect of non-NaCl sodium salt and chloride salt on viral replication, in the presence of ion channel regulators.
3. Whether or not the viral infection could induce HOCl production and NaCl could increase HOCl concentration during infection.
4. If MPO protein is expressed or MPO mRNA is transcribed in our virus permissive cell line.

1.3.1 The reason of choosing RSV, VZV and IAV infection for research.

Previously it has been shown that NaCl could inhibit Herpes Simplex Virus 1 (HSV-1) replication in HeLa cells. We want to look at if NaCl can inhibit the replication of RSV IAV and VZV. RSV is and IAV are both single-stranded, negative-sense genome RNA viruses that could cause VRTI in human (Song et al. 2010). RSV belongs to paramyxoviridae family and is one of the major reasons for hospital visits for children under 2-3 years old. (Song et al. 2010) IAV belongs to orthomyxoviridae family, and could cause influenza in human. (Strengert et al. 2014)

VZV, which closely related to HSV, is a double-stranded linear DNA enveloped α -herpesvirus in herpesviridae family. (Kinchington et al. 2012) Both VZV and HSV share similar replication cycle such as the establishment of latency phase but they are slightly different in the detailed mechanism of replication (Kinchington et al. 2012). By looking at these viruses we aim to explore if the antiviral effect of NaCl is virus-specific or broadly achieved.

1.3.2 Sodium and Chloride Ion channels and preliminary data

According to our hypothesis, cross membrane ion transportation is required because HOCl is produced intracellularly. So the ions from NaCl must enter the cells prior to affecting intracellular pathway. Sodium and chloride ions enter the cell via ion

channels located on cell membrane. Ion channels are pore-forming membrane proteins that gate ion flow across cell membrane. (Catterall et al. 2007) So far there are 2 different sodium channels: voltage-gate sodium channels (VGSC) and ligand-gated sodium channels (LGSC), and 4 different chloride ion channels: cystic fibrosis transmembrane conductance regulator (CFTR), Calcium activated chloride channels (CaCC), Voltage gated chloride channels (ClC), Ligand gated chloride channels (LGCC) (Verkman & Galiotta 2009) (Jentsch et al. 2002). These channels differ from their mechanism and regulate different cell function. Channel inhibitors are a series of chemicals which could compromise ion channel function and prevents ion from entering the cells (Table 2) (Verkman & Galiotta 2009).

Table 2. Ion channels and corresponding inhibitor

Ion Channels	Inhibitor
VGSC	Benzamil (BHH), Ralfinamide
LGSC	No corresponding inhibitor found in current literature
CFTR	CFTRinh172, GlyH101
CaCCs	CaCCinhA01, CaCCinhB01, Tamoxifen
ClCs	5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB)
LGCCs	No corresponding inhibitor found in current literature

1.3.3 Intracellular MPO and HOCl detection

Commonly HOCl could be detected by spectroscopy, electrochemistry, titrimetric and thermochemical method, and fluorescence probe. (Eryilmaz & Palabiyik 2013) HOCl fluorescence probe usually causes minor disturb on cell metabolism and have relative high sensitivity. Such properties make fluorescence probe ideal for intracellular HOCl detection.

HSCe HOCl specific organic fluorescent-probes have been developed by Wu S to

detect intracellular production. (Liu & Wu 2013) HCSe is a red powder with a molecular weight of 441.0495 and dissolve in dimethyl sulfoxide (DMSO). HCSe has an emission fluorescence at 526nm wavelength when excited by light with 510nm wavelength in the presence of HOCl. (Figure 2)

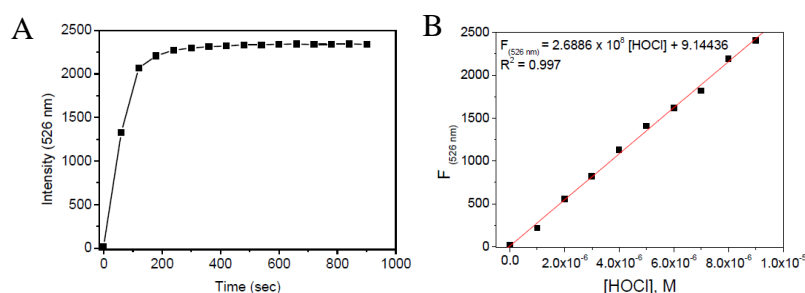


Figure 2. The property profile of HCSe HCSe could reach its fluorescence intensity peak in 300 sec (A) and have a linear relation between HOCl concentration and fluorescence intensity (B). Figure 2 from (Liu & Wu 2013)

1.3.4 Preliminary findings

Previously our research group had achieved a series of results. First, HSV-1 replication in HeLa cells was shown to be inhibited by NaCl in a dose-depended manner. HeLa cells were viable at additional NaCl concentration lower than 100mM, indicates the inhibition of HSV-1 replication was an effect of the salt rather than due to its toxicity. Preliminary research also found a reverse of viral inhibition by NaCl in the presence of chloride channel blocker NPPB. But sodium channel blocker Ralfinamide and BHH did not affect the inhibition of HSV-1 by NaCl. (Wong & Twomey 2014)

2. Materials and Method

2.1 Cell culture

2.1.1 Media and solutions

The cell culture media and solutions used in this project are listed in Table 3.

Table 3. Media and solutions	
Name	Description
DMEM	Dulbecco's Modified Eagle's Medium with 4.5g Glucose/L and L-Glutamine, with phenol red (Lonza BioWhittaker™)
Phenol red free DMEM	DMEM:F12, 1:1 Mixture (Lonza BioWhittaker™)
EMEM	MEM Eagle with Earle's BSS and L-Glutamine, with phenol red (Life technologies)
FCS	Fetal Calf Serum (Life Technologies)
CS	Calf Serum (Life Technologies)
P/S	Penicillin and Streptomycin, final concentration in media: 100 units/ml penicillin and 100µl/ml streptomycin (Life Technologies)
NEAA	MEM Non-Essential Amino Acids Solution (Life Technologies)
PBS	Phosphate buffered saline, 8.0g/L NaCl, 0.2g/L KCl, 1.42g/L Na ₂ HPO ₄ , 0.24g/L KH ₂ PO ₄
HSCe	Powder form HCSe dissolved in pure DMSO to 10mM
Trypsin	0.025% trypsin and 0.01% EDTA in Phosphate Buffered Saline (PBS), with phenol red. (Lonza BioWhittaker™)
Ralfinamide	Powder form, dissolved in water to 25mM (Tocris)
BHH	Powder form, dissolved in ethanol 10mM (Tocris)
NPPB	Powder form NPPB dissolved in ethanol to 5mM (Tocris)
Gel transfer buffer	48mM Tris-base, 39mM glycine, 0.037 SDS, 10% methanol
TBS-Tween	Tris-buffered saline, 10mM Tris-HCl, 100mM NaCl ₂ , 0.2%

	Tween 20
Cell lysis buffer	4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl

2.1.2 Cell lines

Cell line used and corresponding media required are listed in Table 4.

Table 4. Cell lines and required media		
Cell name	Cell type (organ origin)	Growth media
HeLa	Human epithelial cells (cervix)	DMEM+5%FCS+P/S
MeWo	Human fibroblast cells (skin)	EMEM+5%FCS+P/S+1%NEAA
A549	Human epithelial cells (lung)	DMEM+5%FCS+P/S
Vero	Monkey epithelial cells (kidney)	DMEM+5%FCS+P/S
3T3	Mouse fibroblast cells (embryo)	DMEM+5%CS+P/S
293T	Human epithelial cells (kidney)	DMEM+5%FCS+P/S
BHK-21	Hamster fibroblast cells (kidney)	DMEM+5%FCS+P/S

2.1.3 Cell maintenance

Cells were incubated at 37°C with 5% CO₂ in humidified incubator (Thermo) and maintained by passaging every 3-6 days at dilutions of 1:4 – 1:10 as required. Cells were maintained in T75 flasks. During cell passage, media was first removed and rinsed with 10 ml PBS in order to wash off FCS. 2 ml trypsin was added in the flask and incubated at 37°C until cells were detached. Trypsin was neutralized by additional of 8 ml media containing 5% FCS. The cell suspension was gently aspirated up and

down to reach single cell suspension.

2.2 Virus infectivity assay

2.2.1 Virus stock

The viruses used in this research are listed in Table 5.

Table 5. Viruses strain used in this project	
Virus name	Description
Herpes Simplex Virus-1 (HSV-1)	eGFP C12-strain (Arthur et al, 2001)
Varicella Zoster Virus (VZV)	VZV-eGFP-infected MeWo cells (vaccine strain Oka)
Respiratory Syncytia Virus (RSV)	eGFP strain, obtained from Dr. Juergen Schwarz
Influenza A virus (IAV)	A/Udorn/307/1972 (H3N2)

2.2.2 HSV and RSV infectivity in the presence of NaCl assay

HeLa cells were seeded in a black 96-well plate at 1×10^4 cells/100 μ l/well and incubated at 37°C for 24 hr. Viruses stock was diluted to a multiplicity of infection (MOI) of 0.5 in growth media. Supernatant in the well was then removed and replace with 25 μ l virus dilution or 25 μ l growth media only in uninfected control wells. After incubating at 37 °C for 1 hr the wells were emptied by shaking and 50 μ l media (A phenol red free DMEM with 5% FCS and P/S should be used for since phenol red could disturb the fluorescence measurement, this also applied to 2.2.3) was added. Then 50 μ l 2x dose NaCl dilution series (NaCl was diluted by adding 2M NaCl solution into phenol red-free media) in the well. Virus growth was measured in 2 hr intervals between 24 hr to 54 hr where a full growth curve could be collected post-infection as a function of eGFP fluorescence using a POLARstar OPTIMA plate reader (BMG Labtech) with excitation wavelength of 490 nm and emission

wavelength of 520 nm.

Data analysis: The fluorescence value of uninfected cells was removed from all readings as background. Viral replication curve was drawn by plotting fluorescence value against time. The virus replication was calculated by slope value derived from the linear part of viral replication curve. All virus replication under different salt concentration was normalized to the replication with no addition salt. Experiments were carried out in 4 technical replicates.

For RSV, each experiment was carried out in two biological replicates. For VZV, each experiment was carried out in three biological replicates.

2.2.3 VZV infectivity in the presence of NaCl assay

VZV is a cell-associated virus and infection was done with VZV infected MeWo cells. MeWo cells were seeded in a black 96-well plate at 1×10^4 /100 μ l/ well before incubate at 37°C for 24 hr. Frozen VZV stock was defrosted at 37°C and transferred in to a 50 ml falcon tube. 35ml cell growth media was added in the tube dropwise before centrifuging at 1000g for 10min. Cell pellet was resuspended in growth media to a MOI 0.015. Wells were then emptied and 50 μ l 2x concentration virus dilution was added before adding 50 μ l 2x concentration NaCl solution. Virus growth was measured in 2h intervals from 24h to 108h post-infection (where a full growth curve could be achieved) as a function of eGFP fluorescence using a POLARstar OPTIMA plate reader (BMG Labtech) with excitation wavelength of 490 nm and emission wavelength of 520 nm. VZV infectivity data was analysed with the method mentioned in 2.2.2. Each experiment was carried out in four technical replicates.

2.2.4 Sodium and chloride channel blockers assay

HeLa cells were seeded in a black 96-well plate at 1×10^4 /100 μ l/ well before incubating at 37°C. After 24 hr, a dilution series of 1 μ M, 2.5 μ M, 5 μ M, 10 μ M 20 μ M and 40 μ M of channel blocker was prepared by diluting the channel blocker stock with growth media. Media was replaced with 100 μ l channel blocker series or growth media

as control and incubated for 24 hr. Virus stock was diluted to MOI 0.5 in phenol red-free growth media (see 2.2.2). Wells were then emptied and replaced with 25 μ l virus dilution, or growth media as control before incubating at 37°C for 1 hr. Wells were then emptied by shaking and 50 μ l 2 x concentration channel blocker dilution series or media as control were added in the same order. Then 50 μ l 2x concentration salt dilution series were added in the well. Virus growth was measured in 2h intervals between 24h to 54h post-infection (where a full growth curve could be collected) as a function of eGFP fluorescence using a POLARstar OPTIMA plate reader (BMG Labtech) with excitation wavelength of 490 nm and emission wavelength of 520 nm. Data analysis: The fluorescence value of uninfected cells was removed from all readings as background. Viral replication curve was drawn by plotting fluorescence value against time. Virus replication was calculated by slope value derived from the linear part of viral replication curve. The virus replication in the presence of salt and channel blocker is normalized to the corresponding channel blocker concentration with no salt. Each experiment was carried out in three technical replicates.

2.2.5 IAV infectivity in the presence of NaCl assay

An IAV infection media is pre-made by adding trypsin (1 μ g/ml concentration) in serum-free growth media (DMEM with P/S). A549 cells were seeded in a clear 24 well plate at 1×10^5 cells/ml/well and incubated at 37°C for 24 hr. Influenza virus was defrosted and diluted in IAV infection media at MOI of 0.01. Supernatant in 24-well plate was removed and each well was washed with 1 ml PBS twice before infected with 100 μ l virus dilution for 1 hr. Virus was removed and replaced with salt dilution (dilution was made by influenza infection media). After 12h, viral RNA was purified by the method mentioned in 2.4.1 and analysed by qRT-PCR as described in 2.4.2.

Data analysis: Virus infectivity was measured by IAV nucleoprotein (NP) mRNA quantity. All virus replication under different NaCl concentration was normalized to the replication with no additional NaCl. Each experiment was carried out in two

technical replicates.

2.2.6 Cell viability assay

Cells were seeded in a clear 96-well plate and treated in the same way as described in previous part but without viral infection step. 20 μ l CellTiter-Blue reagent (Promega) was added into each well and incubated at 37°C. Then the plate was read under POLARstar OPTIMA plate reader to collect absorbance value at a wavelength of 600nm.

Data analysis: Cell viability was measured by absorbance value. All cell viabilities under different treatment condition was normalized to cells with no treatment. According to the instruction provided by reagent manufacturer and previous experience, cells with viability above 80% are regarded as viable. Each experiment was carried out in three technical replicates.

2.3 Cellular HOCl detection assay

HeLa cells were infected with HSV-1 as stated in 2.2.2 for 1 hour. Then both uninfected and infected cells were treated with a dilution series of NaCl in media. Wells were emptied and washed with 100 μ l PBS per well 4 hr post infection. HCSe 10mM DMSO solution was then diluted with phenol red-free growth media to 10 μ M. 50 μ l HCSe was added into each well and incubated at 37°C for 10min. Then HCSe was removed and wells were washed with 100 μ l PBS twice. Wells were filled with 50 μ l PBS to prevent cells from drying and HOCl concentration was measured as a function of HCSe fluorescence using a POLARstar OPTIMA plate reader (BMG Labtech) with an excitation wavelength of 490nm and emission wavelength of 520nm. HOCl production level was measured by HSCe fluorescence intensity. All intensity values were normalized to condition that no infection and NaCl is presented.

2.4 RNA purification and qRT-PCR

2.4.1 RNA purification

Cells were washed with 1 ml PBS and 200 µl Trizol reagent was added into each well. Trizol was then collected in a 1.5 ml Eppendorf tube. 40 µl chloroform was added in each tube and incubated at room temperature for 2-3 min, then centrifuged for 15min at 12000g at 4°C. Top aqueous phase (whose volume=V) was then transferred to a new 1.5 ml Eppendorf tube and a mix of 2.5V 75% ethanol, 0.1V 3 M sodium acetate(pH 5.2) and 4 µl glycogen (5 mg/ml)) was added. Mixture was stored at -80°C for more than 2h to precipitate RNA. Then RNA was pelleted down at 12000g for 30min at 4°C and supernatant was removed. White RNA pellet was washed twice by adding 500µl 75% ethanol and centrifuging for 10min at 12000g at 4°C. Pellet was dried and dissolved in 50µl water. RNA concentration and quality was measured by Nanodrop spectrophotometers (Thermo).

2.4.2 qRT-PCR.

A qRT-PCR was performed to measure IAV NP mRNA quantity or to detect MPO mRNA existence. qRT-PCR is a technique using reverse transcriptase to synthesis a cDNA strand, a DNA polymerase to amplify the specific sequence by normal PCR and sequence specific probe to quantify DNA quantity. qRT-PCR was done by Verso 1-step RT-qPCR Mix, low ROX kit (Thermo Fisher) according to manufacturer's instruction. A Master Mix, containing 5 µl 2X 1-Step qPCR Low ROX Mix, 0.1 µl Verso Enzyme Mix, 0.5 µl RT enhancer, 0.8 µl primer and 0.1 µl probe. Then 3.5 µl RNA samples (20 ng in total) was mixed with a Master Mix and cycled as the following temperature protocol: 50 °C for 10 min, 95 °C for 1 min, the a segment of 95 °C 10 s and 60 °C was cycled for 40 times. The probe fluorescence was collected by the end of each cycle. Primer and probe information could be found in Table 6. RNA quantity was measured by cycle threshold (Ct) value. Ct is defined as the number of cycles required for the fluorescent signal to cross the threshold.

The quantitative calibrator reaction was the amplification of housekeeping gene Hypoxanthine-guanine phosphoribosyl transferase (HPRT) 1 gene. HPRT gene have a constant expression level in spite of cell condition. Such property of HPRT makes it useful as a ‘ruler’ to calibrate the quantity of other genes.

Data analysis: For IAV assay, Ct values of both HPRT and NP gene was collected. NP gene quantity was first normalised to corresponding HPRT quantity. Then all NP gene quantities were normalised to the quantity of cells with no NaCl treatment.

For MPO mRNA detection assay, Ct values were used as a qualitative indicator of MPO mRNA expression. No Ct value means no visible MPO mRNA amplification.

Table 6. Primer and probe used in qRT-PCR UPL: Universal Probe Library

Gene name	Primer sequence	Probe number
HPRT	Forward 5'-tgacctgattatttgcatacc-3'	UPL #73
	Reverse 5'-cgagcaagacgttcagtcct-3'	
NP	Forward 5'-gtgcaaagaaacctccatt-3'	UPL #48
	Reverse 5'-gccctcatgtctgaggttct-3'	
MPO	Forward 5'-aagctccgggatggtgat-3'	UPL #81
	Reverse 5'-gatccggggcaatgagat-3'	

2.5 Western blot analyses of MPO protein

Western blot analysis was performed to check if MPO is expressed in our major cell lines (HeLa, A549, MeWo, Vero, 3T3, 293T and BHK-21). Cells were seeded in a 24-well plate at 10^5 cells/ml/well for 24 hr and denatured in 100ul Laemmli lysis buffer at 80°C for 10min and loaded onto 12% SDS-polyacrylamide gel. Proteins were electrophoresed at 150 V for 2 hr. Then proteins were transferred from SDS-polyacrylamide gel to 0.45µm nitrocellulose membranes with 35V for 2 hour. The membrane was then blocked with 5% milk/TBS-Tween for 1 hour. MPO antibody (Monoclonal Mouse IgG2B Clone # 392105, R&D Systems) was diluted 1:6000 in 5% milk/TBS-Tween and applied over the membrane overnight while gently shaking. The antibody was collected in a falcon tube for recycling. Then the

membrane was washed with TBS-Tween for 3x15 min. HRP-conjugated-mouse anti-mouse IgG was diluted 1:3000 in 5% milk/TBS-Tween and applied over the membrane for 1 hr and washed with TBS-Tween for 3x15 minutes each. 2ml of a 40:1 solution of enhanced chemiluminescence (ECL) reagents was made up and applied over the membrane evenly for five minutes. ECL was observed by medical X-ray film (Fujifilm) with an exposure of 5 min, developed with OPTIMAX X-ray film processor.

2.6 RSV virus stock production

2.6.1 RSV virus inoculation

A monolayer of Vero cells was prepared in a T175 flask at 37°C. Then 4 ml RSV dilution was added at MOI of 0.1 in the flask and incubated for 1 hr and replaced with cell growth media. After 2-3 days cells were dislodged with a cell scraper and homogenized in a glass homogenizer. Cell debris was removed by centrifugation. Supernatant was filtered with a 0.45 µm filter and aliquoted before stored in -80°C freezer.

2.6.2 RSV virus titre measurement.

HeLa cells were seeded in a 24 well plate at 1×10^5 /ml/well and incubated at 37°C for 24h. Virus was diluted 3 fold up to $1/3^6$ ($1/2187$) dilution. The plate was infected with virus dilution series at 100 µl per well for 1 hr before supernatant was removed and replace with 1 ml HeLa cell media with 0.5% agarose in liquid form. Cells were fixed with 4% formaldehyde and stained with 0.5% Toluidine Blue O solution when virus plaque is visibly formed. Plaques were then counted and virus titre as pfu/ml was calculated.

3. Results

3.1 NaCl inhibits RSV, VZV and IAV replication

Previously we have introduced that NaCl could inhibit HSV-1 replication in HeLa cells in a dose-dependent manner. However, question remained that if this inhibition effect was universal among other viruses. To test if the effect of NaCl is specific to HSV or if NaCl has a general anti-viral effect, we test the effect of NaCl on RSV replication in HeLa cells, VZV replication in MeWo cells and IAV replication in A549 cells. Cells are infected with viruses and then additional NaCl dilution series or growth media as control were added. Then the effect of NaCl on viral replication is assessed by replication slope of the viral growth curve (RSV and VZV). For IAV, viral replication was assessed by the amount of viral NP protein. Cell viabilities for MeWo cells were also tested to exclude the possibility that the reduction of viral replication is due to the toxicity of NaCl but not the direct effect of NaCl on viral replication.

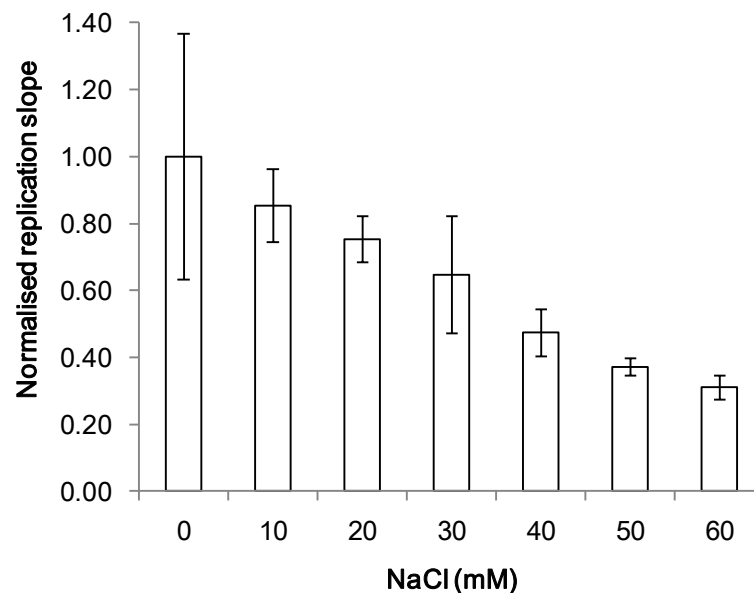


Figure 3. NaCl inhibit RSV replication in a dose dependent manner HeLa cells were infected with RSV at an MOI of 1.0 and treated with a dilution series of NaCl. Virus replication was measured by eGFP fluorescence and replication slope was calculated from the linear phase of growth curve. All viral replications were normalized to that at 0mM NaCl condition. Experiment result is a mean of 3 technical triplicates and representative of three technical replicates. Error bars represent the standard deviation of mean values.

The effect of NaCl on the RSV replication in HeLa cells was tested. A dilution series of NaCl at the concentration from 10mM to 60 mM were used as experiment set. A dose-dependent virus replication inhibition was observed with the increasing concentration of NaCl (Figure 3).

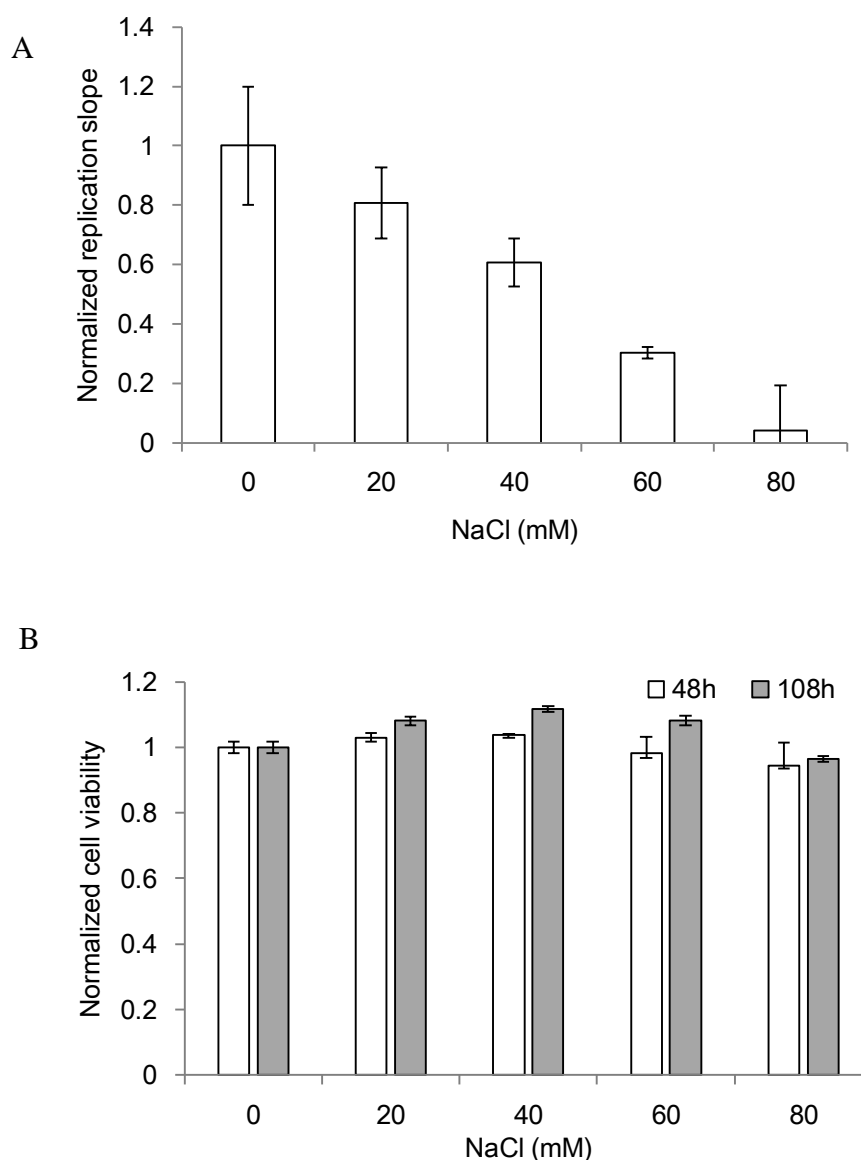


Figure 4. NaCl inhibit VZV replication in a dose dependent manner The effect of NaCl on viral replication (a) and on cell viability (b) were tested. MeWo cells were infected with VZV at an MOI of 0.015 and treat with a dilution series of NaCl. Virus replication was measured by eGFP fluorescence and replication slope was calculated from growth curve. All viral replications were normalized to that at 0mM NaCl condition. Results are an average of 4 technical replicates and representative for biological triplicates. Error bars represent the standard deviation of mean values. For cell viability assay result, values are a mean of technical triplicates normalized to untreated cells. Error bars represent the standard deviation of mean values.

The effect of NaCl on the VZV replication in MeWo was tested at NaCl concentration from 0mM to 80mM. Experiment showed that NaCl inhibit viral replication in a dose-depended manner. Viability test suggested that all NaCl concentration tested showed no obvious cell toxicity which means the inhibition is an effect of salt itself rather than the toxicity. (Figure 4)

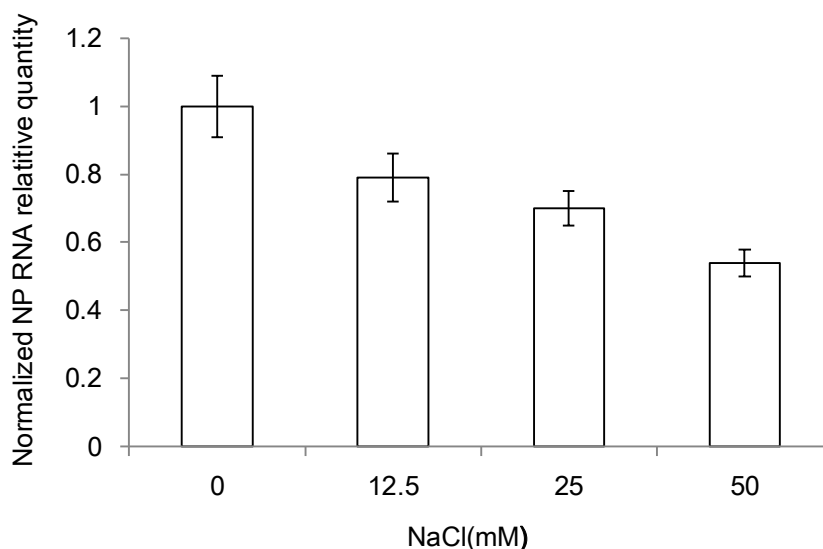


Figure 5. NaCl inhibit IAV replication in a dose dependent manner A549 cells were infected with IAV at an MOI of 0.01 and treated with a dilution series of NaCl. Cells was lysed 12 hr post-infection with Trizol reagent and viral NP RNA was quantified with qRT-PCR. Virus replication was measured by viral NP RNA quantity. All viral replications were normalized to that at 0mM NaCl condition. Error bars represent the standard deviation of mean values from two technical replicates.

The effect of NaCl on the IAV replication in A549 was tested at NaCl concentration from 0mM to 50mM. Experiment showed that NaCl has a inhibition effect on IAV replication in a dose-depended manner (Figure 5).

3.2 The effect of channel inhibitors on viral replication by NaH_2PO_4 and NH_4Cl

Preliminary data showed a reverse of viral inhibition effect by NaCl was observed, in the presence of chloride channel blocker NPPB but not sodium channel blocker Ralfinamide and BHH. Here we design a series of experiments to test if chloride or

sodium channel inhibitor could restore viral replication in other chloride or sodium salt. Sodium ion and chloride ion were separately tested with corresponding channel blocker using 15mM NaH_2PO_4 and 10mM NH_4Cl was used since 50% inhibition of viral replication at such concentration was shown.

3.2.1 Sodium channel blocker Ralfinamide and BHH did not affect the inhibition effect by NaH_2PO_4 .

First we look at the effect of sodium salt NaH_2PO_4 on HSV-1 infection at an MOI of 0.5. Result showed that NaH_2PO_4 inhibit viral replication from 5mM to 40mM concentration. We estimated that 7mM NaH_2PO_4 is required for 50% inhibition of viral replication and 15mM NaH_2PO_4 for 20% inhibition of viral replication. (Figure 6)

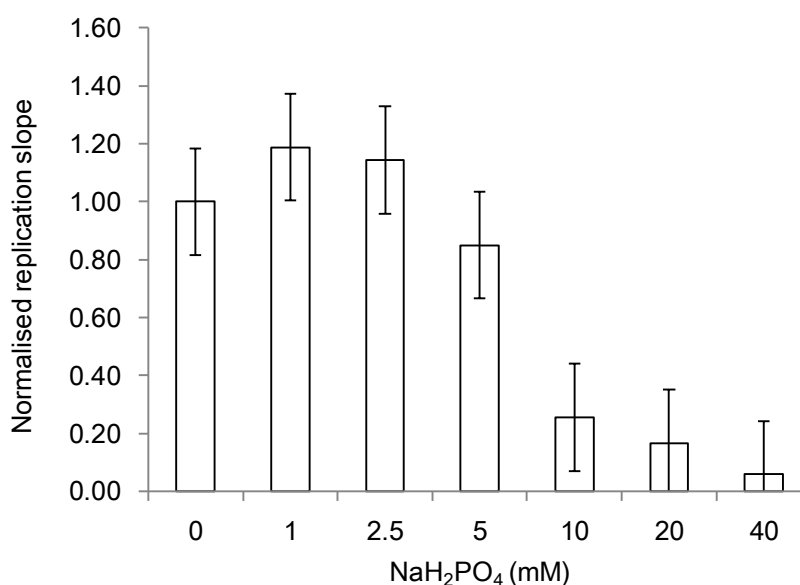


Figure 6. NaH_2PO_4 inhibit HSV-1 replication in a dose-dependent manner from 5mM to 40mM Cells were infected with HSV-1 at an MOI of 0.5 and treat with a dilution series of NaH_2PO_4 . Virus replication was measured by eGFP fluorescence and replication slope was calculated from growth curve. Replication slopes are an average of technical triplicates and normalized to the slope with no salt. Error bars represent the standard deviation of mean values.

Then we decided to look at the effect of sodium channel inhibitor Ralfinamide (inhibit TTX resistant sodium channels) or BHH (inhibit EnaC channels) on the inhibition by NaH_2PO_4 separately. By doing so we hope to find the link between

certain of ion channels regulated the sod HeLa cells were pre-treat with a dilution series of 1 μM , 2.5 μM , 5 μM , 10 μM 20 μM and 40 μM Ralfinamide or BHH, infected with HSV-1 at an MOI of 0.5 and treated with NaH_2PO_4 and Ralfinamide or BHH.

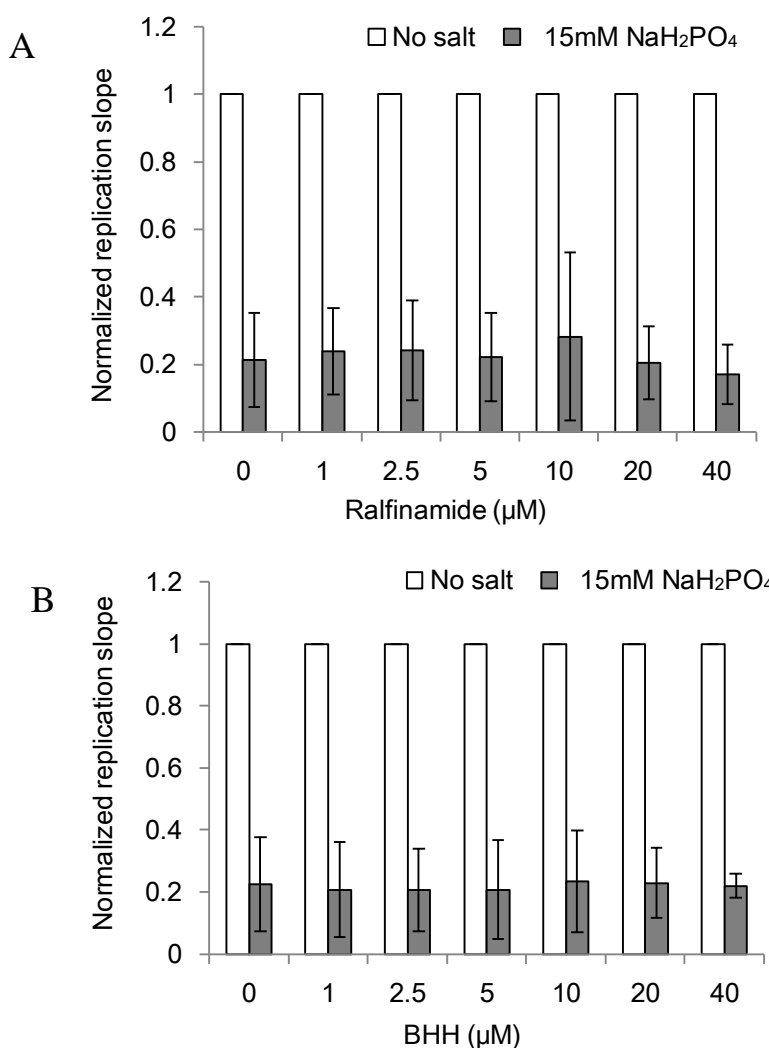


Figure 7. Sodium channel blocker Ralfinamide or BHH did not affect the inhibition of HSV-1 by NaH_2PO_4 . HeLa cells were infected with HSV-1 at an MOI of 0.5 and treat with a 15mM NaH_2PO_4 and a dilution series of Rafinamide (a) or BHH (b). Virus replication was measured by eGFP fluorescence and replication slope was calculated from growth curve. Virus replications in the presence of salt and channel blocker were normalized to those only treated with channel blocker but no salt. Replication slopes are an average of 3 biological triplicates. Error bars represent the standard deviation of mean values.

Result shows a constant inhibition (approximately 20% virus replication) effect by NaH_2PO_4 despite the concentration of Ralfinamide or BHH. We could conclude that

Ralfinamide or BHH did not restore the inhibition of HSV-1 by 15mM NaH_2PO_4 (Figure 7).

Because Ralfinamide and BHH regulates different sodium ion channels, we decided to look if the inhibition of HSV-1 by NaH_2PO_4 could be restored by both Ralfinamide and BHH at each concentration of 1 μM , 2.5 μM , 5 μM , 10 μM 20 μM and 40 μM . We reduce the concentration of NaH_2PO_4 from 15mM to 7mM to see if a restore could be achieved when there was less inhibition of viral replication.

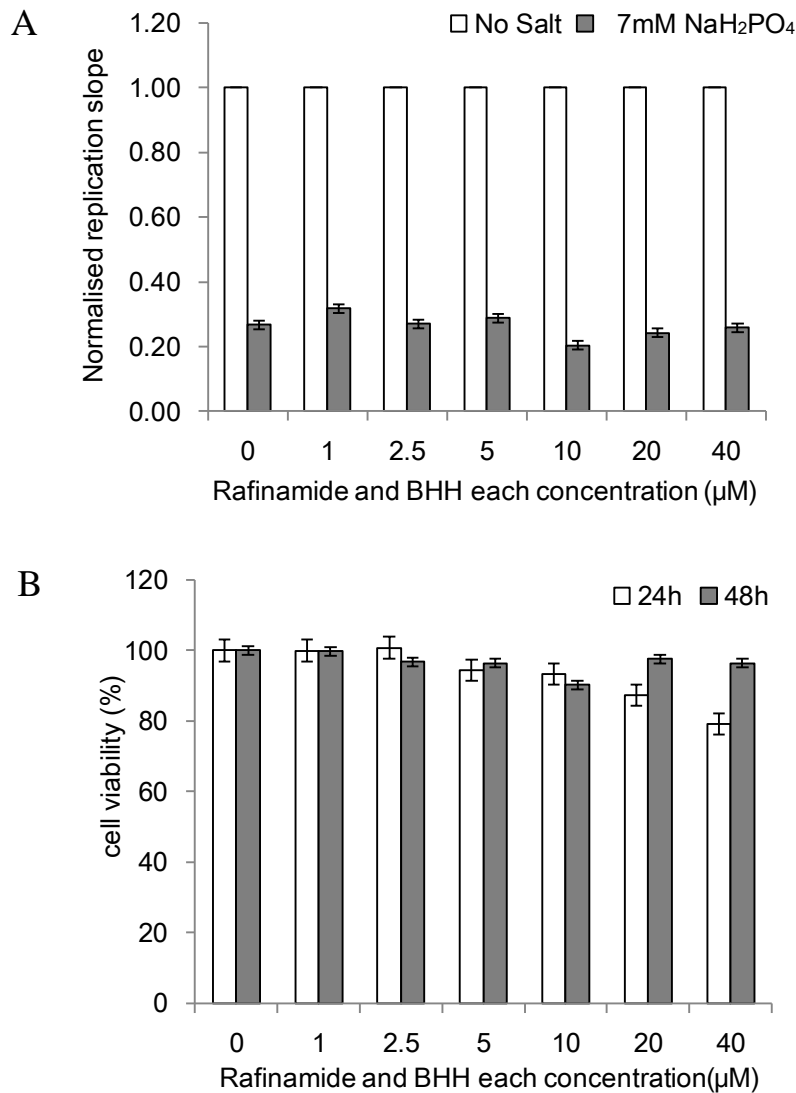


Figure 8. Sodium channel blocker Ralfinamide and BHH together did not affect the inhibition of HSV-1 by NaH_2PO_4 . The effect of Ralfinamide and BHH together on viral inhibition by 7mM NaH_2PO_4 (a) and on cell viability (b) was tested. HeLa cells were infected with HSV-1 at an MOI of 0.5 and treated with 7mM NaH_2PO_4 and a dilution series of Ralfinamide and BHH together. Virus replication was measured by eGFP fluorescence and replication slope was calculated from growth curve. Replication slopes are an average of 3 biological triplicates. Virus replications in the presence of salt and channel blocker were normalized to those only

treated with channel blocker but no salt. Viability experiment result suggested that the result from 40mM Ralfinamide and BHH point is less reliable because cell is less viable (<80%) due to the toxicity of high concentration of channel blockers.

Result shows a constant inhibition (approximately 20% virus replication) effect by NaH_2PO_4 despite the concentration of Ralfinamide and BHH. The result from 40 μM channel blocker concentration is less reliable due to the cell toxicity according to the viability test result. We could conclude that Ralfinamide and BHH together did not affect the inhibition of HSV-1 by 7mM NaH_2PO_4 (Figure 8).

3.2.2 Chloride channel blocker NPPB did not affect the inhibition effect by NH_4Cl

First we look at the effect of chloride salt NH_4Cl on HSV-1 infection at an MOI of 0.5. Result showed that NH_4Cl inhibit viral replication from 0mM to 40mM concentration. We estimated that 5mM NH_4Cl is required for 30% inhibition of viral replication (Figure 9)

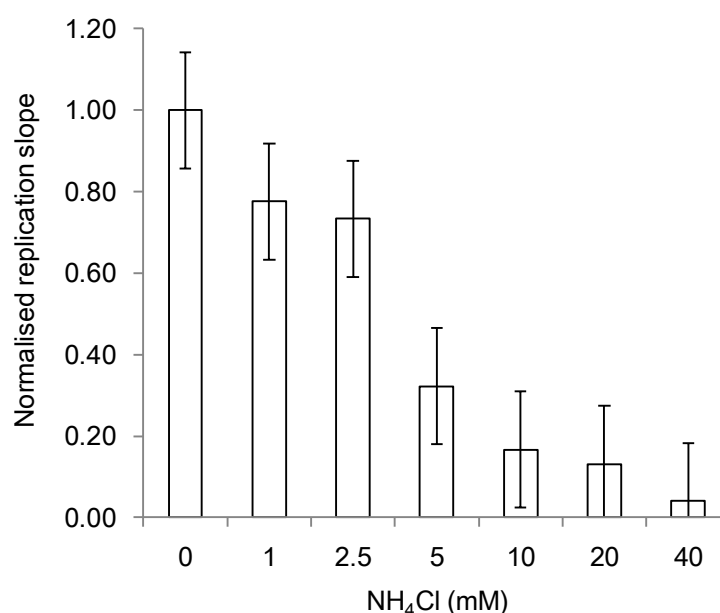


Figure 9. NH_4Cl inhibit HSV-1 replication in a dose-dependent manner from 0mM to 40mM. Cells were infected with HSV-1 at an MOI of 0.5 and treat with a dilution series of NH_4Cl . Virus replication was measured by eGFP fluorescence and replication slope was calculated from growth curve. Replication slopes are an average of 3 technical triplicates and normalized to that with no salt. Error bars represent the standard deviation of mean values.

Then we decided to look at the effect of chloride channel inhibitor NPPB (inhibit

multiple chloride channels) on the inhibition by NH_4Cl . HeLa cells were pre-treat with NPPB dilution series, infected with HSV-1 at an MOI of 0.5 and treated with 10mM NH_4Cl and NPPB dilution series.

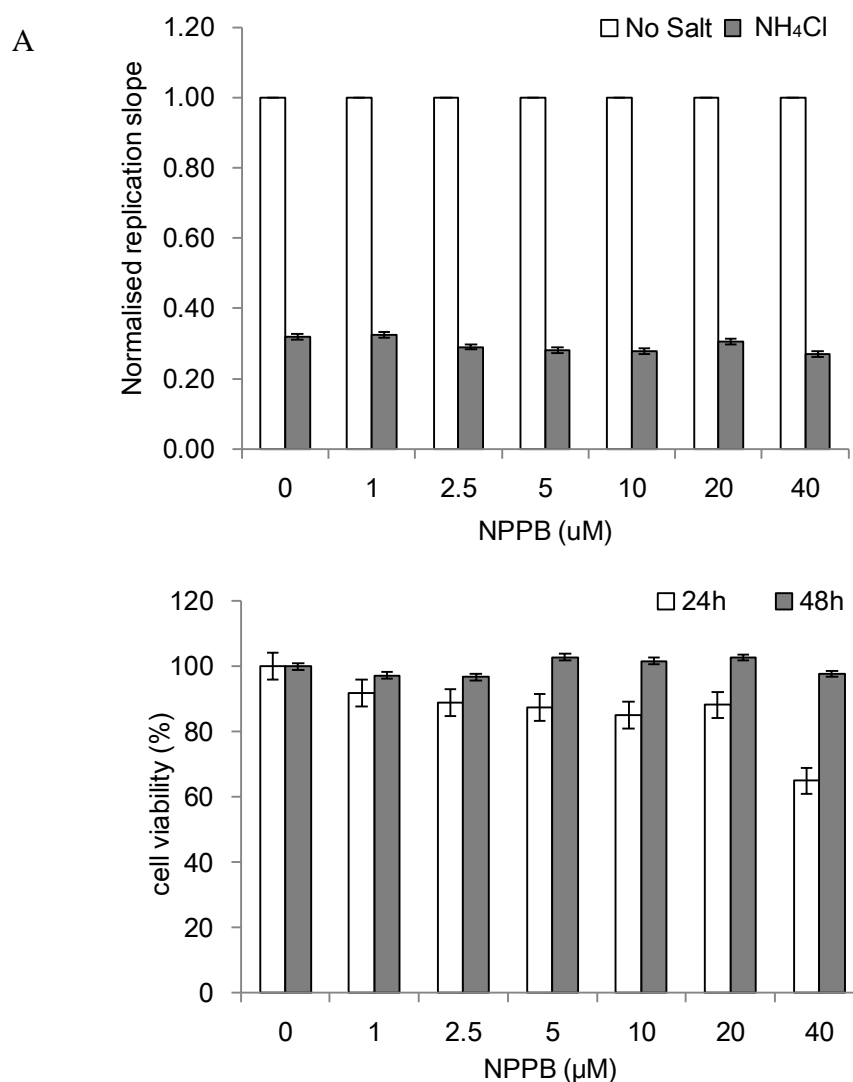


Figure 10. Chloride channel blocker NPPB could not restore the inhibition of HSV-1 by 5mM NH_4Cl The effect of NPPB on viral replication by NaCl (a) and on cell viability (b) were tested. HeLa cells were infected with HSV-1 at 0.5 a MOI and treat with a 5mM NH_4Cl and a dilution series of NPPB. Virus replication was measured by eGFP fluorescence and replication slope was calculated from growth curve. Virus replications in the presence of salt and channel blocker were normalized to those only treated with channel blocker but no salt. Replication slopes are an average of technical triplicates and representative of biological triplicates. Error bars are stand deviations of mean values. For the cell viability assay, values are a mean of triplicates normalized to untreated cells and error bars are standard deviations of mean values.

Result showed a constant inhibition (approximately 30% virus replication) effect by

5mM NH_4Cl despite the concentration of NPPB. We could conclude that Ralfinamide and BHH together did not restore the inhibition of HSV-1 by 7mM NaH_2PO_4 . Viability experiment result suggested that the result from 40mM NPPB point is less reliable because cell is less viable (<80%) due to the toxicity of high concentration of NPPB. (Figure 10)

3.3 Viral infection induce intracellular HOCl production and NaCl increase such HOCl concentration

Our hypothesis is that viral replication could induce intracellular HOCl production, and NaCl could increase the level of HOCl production. To test this, Cells are infected with HSV-1 for 1h and treated with NaCl dilution series post-infection. Intracellular HOCl concentration was measured at 2 h post-infection by HCSe. Another group of cells were treated with same dilution series of NaCl but did not infected with virus as control. HCSe is a fluorescence probe and its fluorescence intensity increase with the level of HOCl.

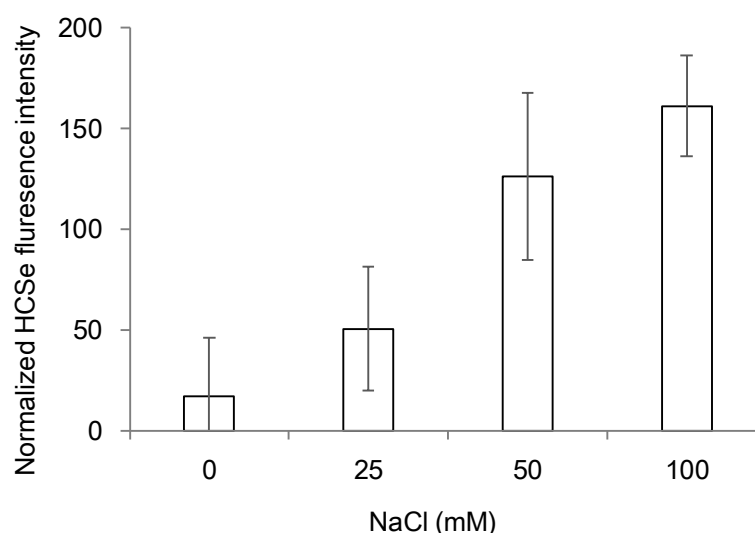


Figure 11. HSV-1 infections induce intracellular HOCl production and NaCl increase HOCl concentration HeLa cells were infected with HSV-1 at 0.5 MOI for 1 h and treat with a dilution series of NaCl. After 2 hr, cells were washed and dyed with 10 μM HCSe for 10min and read under plate reader. The level of HOCl was showed as the florescence intensity of HCSe dye. Fluorescence intensities of infected cells were normalized to uninfected cell with corresponding salt concentration. Result is a mean of three technical replicates. Error bars are standard deviations of mean values.

A production of HOCl was observed when cells were infected with HSV-1, compared with uninfected cells. An increase of HOCl level was seen with the increase of additional NaCl concentration. The HOCl production had a dose-dependent relation with additional NaCl concentration (Figure 11).

3.4 MPO gene mRNA and protein screening in multiple cell lines

The reaction of HOCl production is catalysed by MPO protein in macrophages and neutrophils. We hypothesized that non-phagocytes cell might use the same enzyme to synthesize HOCl. In order to further explore the mechanism of intracellular HOCl production, we did a screen on MPO mRNA and protein in a screen bank consist of HeLa, A459, MeWo, 3T3, Vero, BHK-21 and 293T cells.

Intracellular RNA was separated and purified before analysed by qRT-PCR. MPO mRNA was quantified by Cycle threshold (Ct) value, which is defined as the number of cycles required for the fluorescent signal to cross the threshold. The qRT-PCR showed no Ct values for any cells in our screening.

Then we did a screen on MPO protein in our screen bank. Cellular protein is extracted and separated by electrophoresis. Cellular proteins were washed with MPO antibody and a second anti-MPO antibody with an enzyme ligand which could visualize MPO by producing fluorescence product.

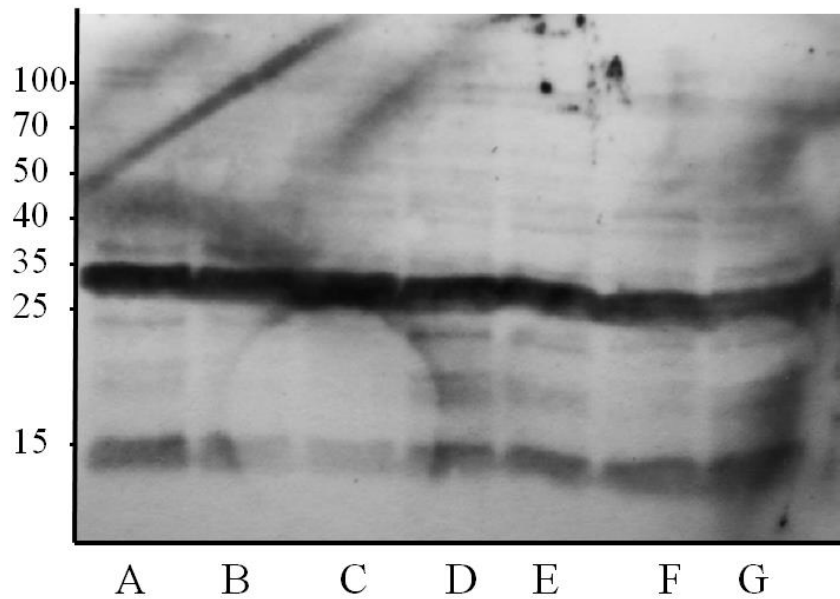


Figure 12. No MPO protein was found during MPO western blot analysis Cells was incubated for 24 hr and cellular protein was collected. Cellular protein was separated by SDS-PAGE. Then protein was stained with MPO antibody and visualized by florescence that catalyzed by enzyme on second antibody. Left marker indicate protein weight by kDa. HeLa (a), A459 (b), MeWo (c), Vero (d), 3T3 (e), 293T (f) and BHK-21 (g) cells are tested for MPO. The existence MPO protein should be a band at 80-90kDa area.

MPO protein has a molecular weight of 80-90 kDa. MPO protein was tested in HeLa, A459, MeWo, Vero, 3T3, 293T and BHK-21 cells. No obvious visible bands were seen around 80-90kDa area as shown in all cell type tested (Figure 12).

4. Discussion and conclusion

4.1 How NaCl broadly inhibit viral replication?

We demonstrate that NaCl could inhibit RSV, IAV and VZV replication in a dose-dependent manner from 0-100mM. NaCl is also inhibitory to Mengo virus between 15mM and 150mM. These results suggest that the inhibition of NaCl is more general phenomenon rather than a viral species specific effect because HSV-1 and VZV are DNA viruses whilst RSV and IAV are RNA viruses. DNA and RNA have significant differences between their replication mechanism, which means NaCl may not target at specific viral enzyme or protein but inhibit virus by blocking general viral replication through another means. There are several possible explanations on the broad antiviral effect by NaCl:

NaCl reduces cell growth A reduction of cell amount was observed in the presence of additional NaCl and more than 50% cell decrease was seen at additional 100mM NaCl concentration (Stubblefieldt 1960). The reduction of cell growth results from the dissociation of the nucleic acid and protein syntheses from cell proliferation. The decrease of host cells by high NaCl concentration might leads to a reduction of viral replication due to the decrease of viral hosts.

NaCl affects cellular metabolism Another possible explanation could be that additional NaCl affects the general cellular metabolism. 100mM NaCl could inactivate DNA polymerase function, affect cell cycle and induce cell apoptosis. (Stubblefieldt 1960) Cells affected by NaCl may no longer be able to maintain a suitable environment for viral replication.

We introduced the viability test in this experiment to look at the metabolism condition of cells. However, while cell viabilities were good (> 80%) during most of our experiment conditions, a decrease of cell viabilities could be seen with the increase of NaCl of channel blockers. This indicates that additional NaCl, though still not cytotoxic, will affect cell metabolism. Also, viability test is a measure of the metabolic ability to change Resazurin (active substance in viability reagent) into

Resorufin. (Promega 2013) We do not know if other metabolic functions, especially those which involve in viral replication, are also fine in those cells with good viability.

NaCl boosts cellular immune response NaCl might inhibit viral replication by boosting immune response. High NaCl pressure could result in more reactive oxidant species (ROS) production in cells (Koga et al. 2008). ROS is responsible for IFN- λ secretion which controls virus infection and additional salt might help to enhance such viral inhibition effect through IFN- λ (Sena & Chandel 2012). High salt presence could also result in elevated type-2 nitric oxide synthase (Nos2)-dependent NO production to facilitate pathogen removal (Jantsch et al. 2015).

Maybe the presence of NaCl may activate certain immune related pathway that lead to virus clearance. We need to test these possible mechanisms individually in future research to conclude how NaCl could broadly inhibit viral replication.

The general antiviral effect by NaCl suggests that NaCl could be a safe and effective antiviral agent, especially in viral infections of epithelial cells during respiratory infections and herpes infections.

4.2 Viral inhibition mechanism by sodium ion and chloride ion.

We initially introduced NaH_2PO_4 and NH_4Cl to look if the antiviral effect of NaCl is due to sodium ion or chloride ion, since NaCl is completely ionized in water and exists as Na^+ and Cl^- . Our results suggest that both sodium ion and chloride ion are inhibitory on viral replication.

Sodium ion Whilst we found that NaH_2PO_4 is inhibitory on viral replication, Spier group's data showed that NaH_2PO_4 did not affect virus infectivity at 150mM (Spier 1960). Such difference could result from that NaH_2PO_4 was mixed with viruses only before and during virus adsorption in Spier group's research, whilst in our research NaH_2PO_4 was presented in the cell culture media during all viral replication period. NaCl is also shown to be not able to inhibit viral replication if only presented with viruses prior to infection. This indicates that the antiviral effect of sodium ion is not a

direct effect on viruses but an effect on infected the cells.

It has been reported that the increase in the intracellular sodium ion concentration by opening sodium ion channel leads to a reduction in IAV replication, whereas a decreased intracellular sodium ion concentration by sodium channel blocker amiloride can boost viral replication. More specifically, the replication of IAV is correlated with the function of $\text{Na}^+/\text{Ca}^{2+}$ exchange channel (Hoffmann et al. 2008) and IAV could inhibit such sodium channels upon infection. These suggest that intracellular sodium concentration is critical for viral infection and virus have developed strategies to affect intracellular sodium concentration in order to survive. So, it is possible that additional sodium ions could increase the intracellular sodium concentration, which leads to a reduction of viral replication.

From clinical aspect, sodium ion transport of epithelial cells is essential for the alveolar fluid clearance (AFC) in RSV infection in respiratory system. AFC could maintain the function of lung during RSV infection. Research found that RSV infection upregulates UTP production and activates protein kinase C (PKC), which reduce the function of ENaC channel and finally compromise the sodium ion transport. Additional sodium ion might be able to restore the sodium ion transport and help maintain the AFC in lung (Song et al. 2010). This may explain why NaCl inhalation is helpful in patients with RSV infection.

Chloride ion While viral infection reduce intracellular sodium concentration, a increases intracellular chloride influx was observed when cells are infected with virus. (Zheng et al. 2014) The viral replication is reduced when chloride ion channel expression is silenced (Igloi et al., 2015). Molecular mechanism investigation reveals that chloride channels CIC-2, CIC-3, CIC-5, and CIC-7 regulates chloride influx (Igloi et al. 2015). Why viral infection could induce the increase of chloride flux? A possible answer is that viral infection might activate HOCl production as an immune response. As chloride ion is an essential substrate for HOCl production, maybe the increase of chloride influx is an immune metabolism change in order to produce more HOCl. If this is the case, additional chloride ions might increase the chloride ion transportation, which finally lead to an increase of HOCl which neutralize the

viral replication.

In summary, both NaH_2PO_4 and NH_4Cl inhibit viral replication which indicates that the antiviral effect of NaCl is a cooperative effect of both sodium ion and chloride ion. The mechanisms inhibitory effect of sodium and chloride ion is different from each other. However, we still need to check the potential antiviral effect of monophosphate and ammonium in viral replication before we firmly draw the conclusion that both sodium and chloride ions are antiviral, from these NaH_2PO_4 - NH_4Cl experiments.

4.3 Why Ralfinamide, BHH and NPPB did not show any obvious effect on viral inhibition by salts?

We introduced channel blocker Ralfinamide, BHH and NPPB to look at the role of ion entry in viral replication. While NPPB reverse the viral inhibition by NaCl in preliminary experiment, no obvious effect on viral inhibition by NH_4Cl was seen. Sodium ion channel blockers Ralfinamide and BHH also showed no effect on viral inhibition by NaCl and NaH_2PO_4 . These results are contrary to conclusions from both our hypothesis and literature reviews that ion transportations are essential for viral replication. Here are some possible explanations for such controversial result.

Sodium ion entry Ralfinamide and BHH regulate tetrodotoxin-resistant Na^+ currents and ENaC channel respectively. So maybe these two sodium ion channels might have minor effect on viral replication. Other research results show that $\text{Na}^+/\text{Ca}^{2+}$ exchange channel have more correlation with IAV replication compared with other sodium channels (Hoffmann et al. 2008). So it seems that different types sodium channels are not equally contribute to the intracellular sodium concentration which affects viral replication. Maybe those sodium channels that regulate HSV-1 infection are not affected by Ralfinamide and BHH. An alternative sodium channel blocker that block other sodium ion channels, apart from tetrodotoxin-resistant Na^+ currents and ENaC channel, might be able to affect the viral inhibition by sodium ions.

While we introduce NaH_2PO_4 as an alternative sodium salt to see the effect of

sodium ions on viral replication, we ignored the potential antiviral effect of monophosphate ions. Ralfinamide and BHH were not being able to affect the antiviral effect by NaH_2PO_4 if monophosphate could inhibit viral replication. Though no evidence was found so far that monophosphate ions are antiviral in literature, we still need to check the effect of monophosphate on viral replication to conclude that sodium ions are antiviral.

Chloride ion entry No restoration of viral inhibition is seen when chloride ion channels were blocked in NH_4Cl experiment. This is controversial to our group's preliminary finding that NPPB restore the inhibition on virus by NaCl . A possible explanation could be that NH_4Cl has its own genuine antiviral effects from NH_4^+ : NH_4Cl could inhibit the envelopment of viral nucleocapsids by disturbing the pH of an intracellular compartment, which is crucial for viral envelopment.(Koyama & Uchida 1989) NH_4Cl also inhibits Human Papillomavirus (HPV) infection by inhibit cysteine proteases, a critical cellular enzyme required for HPV infection (Dabydeen & Meneses, 2009). It could be these unique properties of NH_4Cl that make inhibition profiles of two salts look different. So chloride channel blocker will not be able to reverse such inhibition by NH_4Cl .

It should be noticed that it is hard to establish the connection between one single ion channel to viral infection, as different intracellular ion transportations are tightly linked to each other. For example, calcium ion regulates $\text{Na}^+/\text{Ca}^{2+}$ exchange channel, thus also affect sodium ion transport. As many of channel blockers can target more than one type of ion channel, it become hard to find the connection between one specific ion channel and viral replication by only using channel regulators (Igloi et al. 2015). Also, ion channel regulators not only affect ion transportation but also bring other cellular effect. It has been reported that some ion channel blockers could induce either a pro- or anti-apoptotic state (Hoffmann et al. 2008), which will affect viral replication. We still need more investigation before we linked the results from channel blocker assay to the effect of ion entry on viral replication.

4.4 The mechanism of HOCl production in epithelial cells

HOCl has been found to be produced by human neutrophils and macrophages to neutralize pathogens.(Chapman et al. 2009) Here we have demonstrated that HOCl is produced in HeLa cells in a few hours post viral infection. This indicates that HOCl production in epithelial cells is an early and quick defence response.

It is still unclear about the pathway and mechanism of such HOCl production in epithelial cells. In neutrophils, the HOCl production requires hydrogen peroxide and the catalytic activity of myeloperoxidase and regulated by a few intracellular substances such as immunoglobulin G (IgG) and calcium ions. (Klebanoff 2005) We are still looking if epithelial cells also produce HOCl in similar pathway or have its own separate mechanism.

4.5 How does NaCl help in intracellular HOCl production?

NaCl is shown to increase intracellular HOCl concentration in epithelial cells. There are several mechanisms lead to such phenomenon: NaCl could provide chloride ion as substance to produce HOCl, and this hypothesis fits the fact that chloride flux increased during viral infection. (Klebanoff 2005) An increasing chloride influx might be an immune strategy to bring more intracellular chloride ions as substrate to synthesize HOCl. Another explanation is that NaCl boost HOCl production by increasing ROS level. ROS is a group of chemically reactive molecules containing oxygen and produced by cell naturally as a byproduct of metabolism. (Reshi et al. 2014) ROS are sometimes produced to eliminate pathogen by oxidation of viral protein and nucleic acid. (Lipinski et al. 2009) Since HOCl is also a kind of ROS, it could be produced along with other ROS in the presence of additional NaCl. Other ions such as calcium have been shown to boost DUOX-dependent Reactive oxygen species (ROS) production in $G\alpha_q$ -PLC β -Ca²⁺- DUOX-ROS signalling pathway. (Ha et al. 2009) Intracellular cellular ROS level in the presence of infection and NaCl could be measured in future experiment to test this hypothesis.

4.6 Improvement of MPO detection method

Our hypothesis is that HOCl production in HeLa cells is catalysed by MPO, as mentioned in 4.4. So far we have little evidence about the existence of MPO in HeLa cells. The western blot of MPO protein and qRT-PCR on MPO mRNA showed no existence of MPO protein or mRNA. However, no positive controls were introduced in both experiments so we do not know whether our negative result is genuine or our experiment design and condition was not good enough for detecting MPO.

A good positive control could be HL-60 (human promyelocytic leukemia cells) could be introduced to MPO detection experiments. HL-60 could differentiate in to neutrophil cells which are known to produce MPO and MPO protein has been purified from large-scale HL-60 cell culture. (Hope et al. 2000) MPO protein western blot and mRNA qRT-PCR needs to be repeated with HL-60 as positive control to confirm if MPO is produced in HeLa cells in future experiment.

Apart from western blot and qRT-PCR experiment, MPO gene knockdown assay could also be used to determine the role of MPO in HOCl production. We plan to introduce a MPO knockdown assay by RNA interference. RNA interference (RNAi) is a means of silencing genes by way of mRNA degradation with small interfering RNAs (siRNA). If HOCl concentration decreases when MPO mRNA is silenced, chances are high that MPO regulates HOCl production intracellularly.

4.7 Future work

We observe some inconsistency between each VZV replicates and NaCl cannot inhibit VZV replication until 50mM in some experiments. VZV is a cell-associated viruses and lack of some glycoprotein compared with HSV-1. The mechanism of latency formation for VZV is also different from HSV-1. So we do not know yet whether such inconsistency is due to these differences between VZV and other viruses or due to the error during experiment manipulation such as peppeting. More replicates of VZV are required to understand if NaCl inhibits VZV replication in a different manner.

We cannot be sure if what we observed from these two salts could represent the role of sodium ion and chloride ion in the viral inhibition by NaCl as the role of monophosphate and ammonium in viral replication is not tested yet. Only by ruling out the possible effect by monophosphate and ammonium could we reach a firm conclusion.

During intracellular HOCl concentration experiments we met some inconsistency of the experiment result. We found that the fluorescence of HCSe will increase with light exposure which compromised the accuracy of HOCl result at the early stage of our experiment. Though 96-well plates with HCSe probe were put in light sealed box when transferring between equipment, HCSe might still be affected by light during diluting or pipetting. Improvement such as reduce environment light intensity or use alternative HOCl probe, should be introduced to prevent the interference of fluorescence by light.

Whilst HCSe assay provide information on qualitative information on HOCl production, we desire to quantify the HOCl concentration in cells in order to compare the HOCl level with cell method such as cell flow cytometry. This will help us to compare the HOCl production between epithelial cells and neutrophil cells, whose HOCl production mechanism is known.

We do not know yet the mechanism on how NaCl boost HOCl production. We will look at whether NaCl activates the signalling pathway which lead to HOCl production.

Intracellular MPO protein and mRNA detection assay is not persuasive due to the lack of positive control. HL-60 cells (Human promyelocytic leukemia cells) will be used in future experiment as positive control to help us find out if MPO exist in HeLa cells. SiRNA knockdown of MPO gene could also be used to see if MPO regulate the HOCl production in HeLa cells.

4.8 Conclusion

Our experiment results show that NaCl could inhibit RSV, VZV and IAV replication. Both sodium and chloride ion contribute to such inhibition effect by NaCl. However, the association between sodium and chloride ion entry and viral replication is still unclear due to that channel blockers cannot specifically target at single ion channel.

We found that viral infection could induce HOCl production in HeLa cells, and the presence of NaCl could increase such HOCl concentration. HOCl is produced by MPO in neutrophil cells but if MPO also regulate HOCl production in epithelial cells remain to be discovered.

This research suggests that NaCl have a broad antiviral effect and could be used as general antiviral agent, especially in respiratory infections. Also, we found that HOCl production in epithelial cells is a genuine antiviral immune response.

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